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Influenza H5N1 Virus of Birds Surrounding H5N1 Human Cases Have Specific Characteristics on the Matrix Protein

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The H5N1 influenza virus in Indonesia has caused more than 100 people died due to the virus infections. Cases in humans were mostly due to the virus spread from the infected birds. This study characterized molecularly the H5N1 virus from birds around the H5N1 infection cases in humans in Indonesia. Result from this study revealed that in several cases, waterfowl species could become the source of H5N1 infections in human. We found that the one of six viruses used in this study probably was a first antigenic shift virus in Indonesia. This study shows that the AI viruses isolated from birds around humans infected by H5N1 virus has specific characteristics namely the presence of several amino acid substitutions especially on the M1 and M2 proteins. The substitutions are similar in most of H5N1 human cases in Indonesia.

Key words: molecular character, H5N1 virus, birds, surrounding, human H5N1 infection

INTRODUCTION

The highly pathogenic avian influenza (HPAI) H5N1 virus was known for the first time in 1997, when the virus was directly transmitted from birds to human in Hong Kong and caused 18 respiratory disease cases including 6 people died due to the disease (Claas *et al*. 1998; Suarez *et al*. 1998; Shortridge *et al.* 1998; Bender *et al*. 1999; Katz *et al*. 2000; Ungehusak *et al*. 2005). Then at the beginning of 2003, two H5N1 infection cases were identified in Hong Kong (Peiris *et al*. 2004). Since the end of 2003, the HPAI H5N1 has spread up to Central Asia, Europe and Africa, causing endemic disease and death of domesticated birds and wild birds. Until September 2006, approximately 40 laboratories have confirmed the human cases infected by H5N1, and more than 50% of the infections in humans were fatal (Centers for Disease Control and Prevention 2004; Hien *et al.* 2004).

Continuous exposure to the H5N1 virus on humans will increase the possibility of the influenza pandemic in humans. The avian virus can adapt more efficiently in human through the reassortment with other influenza strains in humans (Webster *et al*. 1992; Taubenbarger *et al*. 2005). Several H5N1 infection cases were family clusters; however, it can be stated that the virus transmission from human to human is still very limited (Ungehusak *et al*. 2005). The viral transmission inter human has not yet been proven, thus most cases in humans occurred due to virus spread from infected birds (World Health Organization 2005a). Genetic analysis shows that most of the H5N1 influenza viruses from birds and human in Asia are the Z genotype, similar to the virus first identified in South China (Guan *et al*. 2004; Li *et al.* 2004; Puthavathana *et al.* 2005).

In Indonesia, since the first outbreak in the late 2003, the H5N1 virus has rapidly became endemic (Smith *et al.* 2006; Sedyaningsih *et al.* 2007) and continued to cause sporadic zoonotic transmission to human beginning in July 2005 (Sedyaningsih *et al.* 2007). Avian influenza (AI) is still a serious case requiring attentions considering the number of victims died due to the infection till now. At least, until 27 January 2009 there were 115 people died due to this virus (World Health Organization 2009). The data on the characters and genetic information related to died victims caused by H5N1 virus infections of birds origin in Indonesia are still limited. In this study, we reported the molecular character of viruses isolated from birds around the homes of human cases of H5N1.

MATERIALS AND METHODS

AI Viruses. During 2006-2007, six H5N1 viruses from the avian species arounding human cases infected by AI virus subtype H5N1 have been isolated (Table 1). Field data of the viruses were identified to determine the avian species origin of the viruses that can cause the H5N1 human infection.

The H5N1 viruses were propagated in 9-11 old day embryo specific pathogen free (SPF) eggs (OIE 2000). These six viruses were then further analyzed on four segments namely hemagglutinin (HA), neuraminidase (NA), matrix (M), and nonstructural (NS).

DNA Sequencing and Analysis of Influenza Virus Genes (HA, NA, M, and NS). To identify the genetic

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characteristic of HA, NA, M, and NS of the H5N1 viruses, we conducted sequencing for those genes. The sequences of four genome segments of AI viruses (HA, NA, M, and NS genes) of the six AI viruses of bird origin isolated around cases in humans infected with AI in 2006-2007 were compared with the sequence data of human H5N1 virus available at GenBank (www.ncbi.nlm.nih.gov). The information of the viruses can be seen in Table 1.

The strategy to amplify full length HA was using primer Senne *et al.* (1996) to amplify HA1 region, and HA2 was modified using H5-155F (Lee *et al*. 2001) and NS890 primers published by Hoffman *et al*. (2001). Primers for NA gene was from Komadina (2006, personal communication), while the amplification of M and NS genes used primer Hoffman *et al.* (2001). The PCR products were separated in 1% agarose by electrophoresis and the amplicon was excised and purified using QIAquick gel purification kit (Qiagen). The sequencing method used was direct sequencing using Cycle sequencing kit (BigDye Terminator version 3.1; Applied Biosystem) on Genetyx Analyzer 3130 (Applied Biosystems, USA).

The nucleotide sequencing data obtained in this study were analyzed together with the genetic data available in the avian influenza database (NCBI) based on each gene. The production of multiple alignments each gene and the residue analysis was carried out by using BioEdit version 7 (http://www.mbio.ncsu.edu/BioEdit). Phylogenetic trees were generated by neighbor-joining bootstrap analysis (1,000 replicates) by using the Tamura-Nei algorithm in MEGA version 4 (Http: //www.megasoftware.net). All of the viruses used in this study have been submitted to GenBank (www.ncbi.nlm.nih.gov) with accession number: A/Muscovyduck/Jakarta/Sum106/2006 (GU183453, GU183472, GU183434, GU183414), A/Duck/Jakarta/Slmt306/2006 (GU183454, GU183473, GU183435, GU183415), A/Muscovyduck/West Java/Bks3/2007 (GU183455, GU183474, GU183436, GU183416), A/Ck/Pessel/BPPVRII/2007 (GU183456, GU183475, GU183437, GU183417), A/Ck/Inhu/BPPVRII/2007 (GU183457, GU183476, GU183438, GU183418), A/Ck/Jakarta/DKI-Nurs/2007 (GU183458, GU183477, GU183439, GU183419).

RESULTS

The Avian Species Origin of the Viruses. All of the victims in this study were infected by the A1 viruses after direct contact with sick birds (Table 1). To collect the viruses from the birds correlated with the human AI infection in Indonesia relative difficult. One of the problems is when AI human case accurs, the government is directly to disinfect the location and stamping out the birds before conducting investigation. During 2006-2007, we just isolated six isolates and three of the viruses (A/Muscovy Duck/Jakarta/Sum106/2006, A/Duck/Jakarta/Slmt306/2006, A/Muscovy Duck/West Java/Bks3/2007) were isolated from waterfowl (duck and muscovy duck). In this case, muscovy duck is an infection source for the AI infection in humans. Muscovy duck as the viral reservoir did not show any clinical symptoms until the presence of AI cases in human at that site (FJ and N, Table 1) was known. From cloacal swab samples taken from duck, we detected AI virus subtype H5N1 and then

Table 1. Samples taken from birds surrounding H5N1 cases in humans

Initiais	Age	Sex	Agreed occupation	District	Province	Onset	Outcome	Date death	Status	Human virus code	Notes	Exposure	Urban or no	Animal virus
FJ	9	M	Student	South Jakarta	DKI Jakarta	7-9-06	Died	22-9-06	Confirmed	CDC835	*	Poultry deaths at home	Urban	A/Muscovy Duck/Jakarta/Sum106/2006 A/Duck/Jakarta/Slmt306/2006
RI	14	M	Student	West Jakarta	DKI Jakarta	31-12-06	Died	10-1-07	Confirmed	CDC887	**	Handled sick/dead poultry	Urban	A/Ck/Jakarta/DKI-Nurs/2007
N	12	F	Student	Bekasi	Weat Jakarta	12-1-07	No information	No information	Confirmed	No information	***	Handled sick/dead poultry	Rural	A/Muscovy Duck/West Java/Bks3/2007
D	14	M	Student	Pasir Selatan	West Sumatra	15-3-07	24-3-07	Died	Confirmed	CDC1031	****	Slaughter sick/dead poultry	Rural	A/Ck/Pessel/BPPVRII/2007
Yt	26	M	Paim plantation worker	Indragiri Hulu	Riau	3-6-07	12-6-07	Died	Confirmed	No information	*****	Slaughter sick/dead poultry	Rural	A/Ck/Inhu/BPPVRII/2007

*: The case had contact with sick chickens (his pets) in his household; **: Case had direct contact with bird/poultry (ducks on 24 Dec 06), since 1 month before investigation bird/poultry in environment (owned by nelghbors) began dying and were disposed located near area were cases family caged their birds/poutry; ***: Case had direct contact with sick birds belong to the neighborhood on 7 January 2007; ****: On 4 March 2007, chicken started dying in the neighborhood (brother,s house). On 9 March 2007, case slaughtered and cleaned two sick chickens. The case lives in rural area 9 hours away from Padang; *****: Symtoms: cough, headache, fever.

isolated it. This case showed that reared waterfowl could be a source of H5N1 infection in humans.

Molecular Characteristics of HA Protein. Phylogenetic analysis of HA gene revealed that all of the viruses used in this study belong to group 1 (Figure 1). Based on the amino acid sequences on HA cleavage sites, the six viruses used in this study have multiple basic amino acids indicating that the viruses are highly pathogenic. The four AI viruses used in this study had the substitution on -6 of HA1 protein i.e. R→S and one virus had substitution R→G, while the other one did not undergo a substitution (Table 2). All viruses were analyzed in this study had the preferential binding of sialic acid joined with the sugar chain through α 2,3 linkage as they had glutamic residues at position 222 (at position 226 for H3 virus) and glysine residues at position 224 (at position 228 for H3 virus) that are avian receptors (Stevens *et al.* 2006). Seven potential glycosylation sites of HA1 at amino acid positions 11, 29, 84, 154, 165, 193, and 286 were maintained among human and bird isolates excluding Pessel/BPPVRII/07. Mutation T/S156A at the Pessel/BPPVRII/07 results in the loss of glycosylation sites (Li *et al.* 2004; Chen *et al.* 2006). Glycosylation at the HA plays a role in antigenic variation by masking and unmasking antigenic site. In this study, Jakarta/DKI-Nurs/07 and West Java/Bks3/07 viruses had no glycosylation site at position 165 and the Jakarta/DKI-Nurs/07 virus also had none at position 193. Thus, Jakarta/DKI-Nurs/07 and West Java/Bks3/07 viruses only have 4 and 6 glycosylation sites at the HA1 respectively (supplemental data).

Molecular Characteristics of NA Protein. At the NA gene level, the six viruses used in this study were closely related to the H5N1 human origin virus and different from virus Gs/Gd/96 or HK/483/97 (data not shown). Two viruses from Sumatra, i.e. Riau and West Sumatra, (A/Ck/Pessel/BPPVRII/07 and A/Inhu/BPPVII/2007) have genetic relationship with H5N1 viruses from birds isolated from Riau and West Sumatra such as the A/Ck/IDN/PekenBaru161-11/06, A/Ck/Agam1631-2/06 and A/Ck/IDN/Padang1631-1/06 viruses. Indonesian H5N1 viruses including the six viruses in this study had 20 amino acid deletions in the stalk region at positions 48-68 (data not shown).

Molecular Characteristics of NS Protein. The PDZ-binding motif of NS1 is a new virulence factor of influenza A viruses (Jackson *et al*. 2008). The motif can bind to cellular PDZ-containing proteins involved in host cellular signaling pathways. Human influenza viruses contain different such as RSKV or RSEV and ESEV or EPEV motif belong to avian origin. From this study, four viruses possessed the ESEV motif indicating that the viruses are of avian origin. The two other viruses have another motif. The Inhu/BPPVRII/07 virus showed KSEV motif and the other hand Pessel/BPPVRII/07 virus possessed human influenza motif i.e. RSEV motif.

Multiple alignment analysis on NS1 showed that the deletions of amino acids at positions 80-84 in five isolates (Table 2), excluding the Pessel/BPPVRII/07 virus. The five amino acid deletions also characterize other H5N1 viruses of the Z+, Z, Y, A, B, and C genotypes and may contribute to the increased virulence (Long *et al.* 2008).

Using pair wise alignments the Pessel/BPPVRII/07 virus showed that nucleotide identities between Pessel/BPPVRII/07 or Hong Kong virus and five viruses used in this study were about 86-88% and 84-88% for amino acid, respectively, even though the identity of NS1 among the five viruses used in this study was 97-99% for nucleotide and 95-99% for amino acids sequence (data not shown) and located in same group with HK/497/97 and HK/498/97 and China virus group (Figure 1b). This is very interesting because the virus isolated from indigenous chicken outbreak around the human H5N1 case in Riau Province, Sumatra Island is in one cluster with Indonesian H5N1 virus on the HA, NA, and M gene, but slightly different on NS1 gene, because the virus is similar to Hong Kong viruses especially H3N2 viruses namely HK/497/97 and HK/498/97.

Pessel/BPPVRII/07 and H3N2 Hong Kong viruses have 8 similar amino acids at the positions 22, 70, 81, 114, 127, 207, 215, and 227 (supplemental data). This result demonstrates that Pessel/BPPVRII/07 virus has HA, NA, and M genes belonging to Indonesian H5N1 viruses originated from avian but for NS1 gene belongs to Hong Kong viruses which is a human influenza virus; hence Pessel/BPPVRII/07 virus is probably a reassortment virus.

Molecular Characteristics of M Protein. The six viruses used in this study demonstrated M2 mutation of V27A indicating their resistance to amantadine (data not shown). The AI viruses from birds isolated from around H5N1 human cases possessed specific amino acids similar to amino acid sequence of H5N1 human viruses at the M protein level. The amino acid on the M1 protein namely Ala/A, Lys/K, Ala/A, and His/H at positions 37, 95, 137, and 249 respectively were identified from human-origin of H5N1 virus or from birds around H5N1 human cases at the location. All of the viruses had four specific amino acid excluded Jakarta/DKI-Nurs/07 virus, the virus had R at position 249 replacing H. On the other hand, most of the H5N1 viruses from bird origin had the amino acids composition of Thr/T, Arg/R, Thr/T, and Gln/Q at the above mentioned positions.

On the M2 protein, though it is not as specific as in the M1 protein, several amino acids were possessed exclusively by the bird origin virus surrounding AI cases in humans, which are Tyr/Y, Lys/K, Ile/I, Ala/A, and Phe/F (at positions 8, 18, 19, 27, and 50 respectively) except for the DKI-Nurs/07 virus that had only 2 amino acids from the amino acid motifs above (A and F amino acids) (Table 3).

DISCUSSION

The molecular character of the avian viruses isolated from around H5N1 human cases on the cleavage site region of HA protein showed that the geographical variation in cleavage site motif may occur and it is not related to the virulent changes and infections in humans (Writing Committee of Second World Organization

Figure 1a. Phylogenetic trees of the H5N1 viruses.

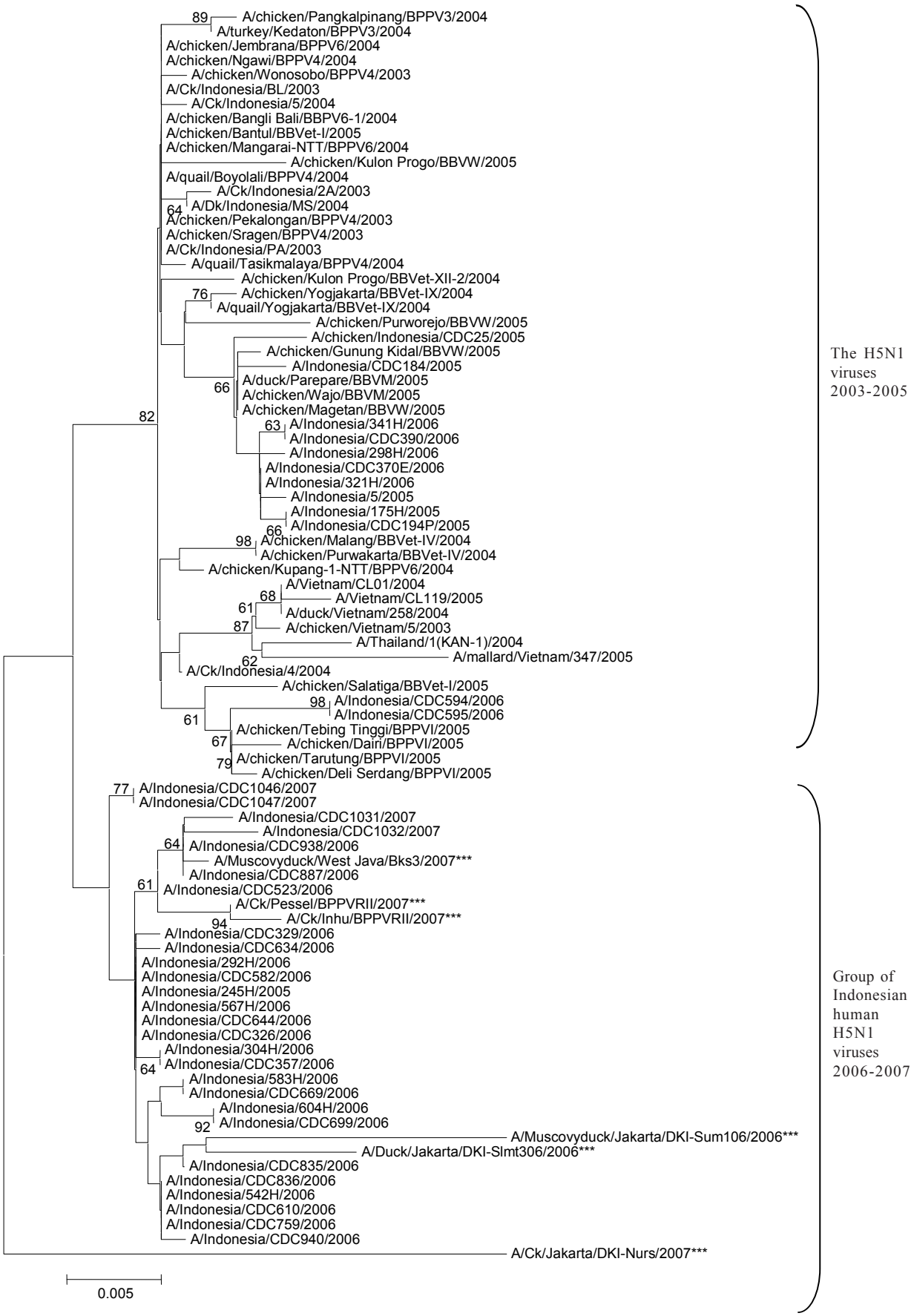

Figure 1b. Phylogenetic trees of the M1 gene.

Figure 1c. Phylogenetic tree of NS1 gene. Phylogenetic trees of the H5N1 viruses. a) Phylogenetic tree of the hemagglutinin (HA), b) Matrix 1 (M1) and c) Non Structural (NS1) gene of the H5N1 viruses isolated from birds surrounding AI human cases in Indonesia. The stars sign showed the viruses used in this study. Nt 49-1680 of HA gene, nt 1-1157 of The NA gene, nt 1-756 of M1 gene and nt 25-690 of NS1 gene were used for the analysis. The construction of phylogenetic using MEGA version 4. A neighbor-joining bootstrap analysis (1,000 replicates) using the Kimura–Nei model is shown.

Table 2. Genetic characters of H5N1 viruses of this study compared with H5N1 human closest isolates from Indonesia in GenBank

Viruses	HA sequence at aa			NS sequence		M 2 sequence at aa	
	222	224	Cleavage site	Deletion of aa 80 to 84	PDZ-binding ligand	27	31
CDC938/2006	Q	G	PQRESRRKKR	YES	ESEV	A	S
CDC835/2006	Q	G	PQRESRRKKR	YES	ESEV	A	S
CDC887/2006	Q	G	PQRESRRKKR	YES	ESEV	A	S
CDC1031/2007	Q	G	PQRESRRKKR	YES	ESEV	A	S
Bks3/2007	Q	G	PQRESRRKKR	YES	ESEV	A	S
Pessel/BPPVRII/2007	Q	G	PQRERRRKKR	NO	RSEV	A	S
Inhu/BPPVRII/2007	Q	G	PQREGRRKKR	YES	KSEV	A	S
DKI-Nurs/2007	Q	G	PQRESRRKKR	YES	ESEV	A	S
Sum106/2006	Q	G	PQRESRRKKR	YES	ESEV	A	S
Slmt306/2006	Q	G	PQRESRRKKR	YES	ESEV	A	S

Table 3. Amino acid prediction at the M gene from H5N1 viruses of avian species from this study correlated with H5N1 human cases

Viruses	Amino acid position of M1				Amino acid position of M2				
	37	95	137	249	8	18	19	27	50
Sum106/2006	A	K	A	H	Y	K	I	A	F
Slmt306/2006	A	K	A	H	Y	K	I	A	F
DKI-Nurs/2007	A	K	A	R	C	R	S	A	F
Bks3/2007	A	K	A	H	Y	K	I	A	F
Pessel/BPPVRII/2007	A	K	A	H	Y	K	I	A	F
Inhu/BPPVRII/2007	A	K	A	H	Y	K	I	A	F
Indonesian H5N1 viruses origin from avian species	T	R	T	Q	C	R	S	V	C
Most Indonesian human H5N1 viruses 2006-2007	A	K	A	H	Y	K	I	A	F

Consultation, 2008). The presence of glycosylation sites at 154-156 from six viruses in this study showed that Z strains of H5N1 isolated since late 2002 in Hong Kong, Indonesia, Thailand, Vietnam, and Yunnan Province, China in late 2003 and 2004 had acquired a potential N-linked glycosylation site at the position. Glycosylation at this site, adjacent to the receptor-binding and antigenic sites at the globular tip of the H5 influenza HA molecules, is capable of altering the receptor-binding and may help the virus to evade the host antibody response (Li *et al*. 2004). Gain or loss of glycosylation sites in HA clearly plays a role in determining morphological characteristics of the virus antigenicity and influences the selection of potential vaccine strains, but the role these sites play in disease is unknown (Bean *et al.* 1985).

Glycosylation sites in the stalk of the neuraminidase play a role in maintaining the tetrameric structure of the protein (Luo *et al.* 1993). All of the isolates from this study have no glycosylation at the stalk of the NA due to the deletion in the stalk region. Deletion in the stalk of the NA is thought to increase the retention of virions at the plasma membrane (Matrosovich *et al.* 1999) to balance weaker binding of sialic acid receptor by the HA with newly acquired N154 glycosylation (World Health Organization 2005b). The isolates in this study have conserved three out of the four glycosylation sites present in the NA at the amino acid positions 88, 145, and 235.

For the NS gene, a large-scale sequencing analysis of avian influenza viruses indicates that the C-termini of avian influenza virus NS1 proteins have the consensus sequence of a PDZ domain ligand (Obenauer *et al.* 2006). They specifically recognize and bind to short C-terminal peptide motifs of 4-5 amino acids (X-S/T-X-V type) at the 227-230 positions of NS1 (Obenauer *et al.* 2006). PDZ ligand binding motifs with ESEV or EPEV sequence were found in the NS1 from HPAI H5N1, H9N2, and H7N7 viruses (Hale *et al.* 2008). The Inhu/BPPVRII/07 virus showed KSEV motif as well as the PDZ-binding motif of 1918 virus and surprisingly, the motif did not belong to avian-like PDZ-binding motif. The KSEV motif was rarely found in nature, and in 2005 it was recorded that two of the H5N1 Indonesian viruses have that motif i.e. A/chicken/Indonesia/CDC24/2005 (Acc. number of GenBank CY014196) and A/chicken/Indonesia/CDC25/2005 (Acc. number CY014189) and Arabian H5N1 viruses in 2007 (Monne *et al.* 2008). On the other hand Pessel/BPPVRII/07 virus possessed human influenza motif i.e. RSEV motif. It is not known whether the motif in Inhu/BPPVRII/07 and Pessel/BPPVRII/07 viruses affects the virulence or adaptation of the virus in human. *In vitro* and *in vivo* studies are required to answer this question.

Several studies reported that NS1 protein is also associated with the virulence and host range of influenza viruses in different animal models (Seo *et al.* 2002; Quinlivan *et al.* 2005; Solorzano *et al.* 2005; Li *et al.* 2006). The Pessel/BPPVRII/07 virus is the virus without deletion at NS1 and like other Indonesian H5N1 viruses did not undergo mutation at position 92; they have aspartic acid instead of glutamic acid at position 92 of the NS1 molecule. The glutamic acid at position 92 of the NS1 protein of the H5N1 influenza virus transmitted to human in 1997 becomes critical in conferring virulence and resistance to antiviral cytokines in pigs (Seo *et al.* 2002). However, H5N1 virus with this amino residue is no longer circulating in nature and glutamic acid is not found in the NS1 proteins of other influenza A virus subtypes. In addition, it was recently reported that a deletion of amino acids at positions 80-84 in NS1 enhances the virulence of H5N1 viruses (Long *et al.* 2008).

This study shows that two viruses of chicken origin (Pessel/BPPVRII/07 and Inhu/BPPVRII/07 viruses) isolated surrounding H5N1 human cases have NS1 unique genetic character which may correlate with the adaptation of the viruses in human. We recommend to conducting the integral surveillance in Riau due to unique viral characteristic from viruses isolated in 2007 and continue to compare between human and animal viruses genetic information and animal experiment for understanding the pathogenicity and virulence of the viruses to human.

From the Matrix protein analysis, most of the virus from human-origin have the above five amino acid motifs, and had at least two of the amino acids and only a few had other motifs. These findings are in agreement with the previous result suggesting that at the M gene level, the human and animal origin viruses differ in the presences of substitutions in M1 and M2 (Chen *et al.* 2006). From the results obtained in this study, it was assumed that there were visible differences between virus from birds that may or may not infect humans. Unlike in M1 and M2 proteins, a specific difference between the viruses that could or could not infect humans was not found in the surface protein genes of H5N1 viruses. However, the motif in M protein was specifically found only in the viruses from Indonesia, not in the other country. This result is in line with Bender's *et al.* (1981) in his study that stated that the analysis of the surface protein genes of the H5N1 viruses from Hong Kong 1997 outbreak did not provide immediate answers regarding the molecular basis for virulence. Even though this study indicates that M1 and M2 proteins may have important roles in predicting whether or not an AI virus can infect humans, in vitro and in vivo studies are still required to answer the question about the role of M protein as a marker for predicting whether or not a virus is capable of infecting human.

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